

Metabolic engineering of essential oil yield and composition in mint by altering expression of deoxyxylulose phosphate reductoisomerase and menthofuran synthase

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Peppermint (*Mentha × piperita* L.) was independently transformed with a homologous sense version of the 1-deoxy-D-xylulose-5-phosphate reductoisomerase cDNA and with a homologous antisense version of the menthofuran synthase cDNA, both driven by the CaMV 35S promoter. Two groups of transgenic plants were regenerated in the reductoisomerase experiments, one of which remained normal in appearance and development; another was deficient in chlorophyll production and grew slowly. Transgenic plants of normal appearance and growth habit expressed the reductoisomerase transgene strongly and constitutively, as determined by RNA blot analysis and direct enzyme assay, and these plants accumulated substantially more essential oil (about 50% yield increase) without change in monoterpene composition compared with wild-type. Chlorophyll-deficient plants did not afford detectable reductoisomerase mRNA or enzyme activity and yielded less essential oil than did wild-type plants, indicating cosuppression of the reductoisomerase gene. Plants transformed with the antisense version of the menthofuran synthase cDNA were normal in appearance but produced less than half of this undesirable monoterpene oil component than did wild-type mint grown under unstressed or stressed conditions. These experiments demonstrate that essential oil quantity and quality can be regulated by metabolic engineering. Thus, alteration of the committed step of the mevalonate-independent pathway for supply of terpenoid precursors improves flux through the pathway that leads to increased monoterpene production, and antisense manipulation of a selected downstream monoterpene biosynthetic step leads to improved oil composition.

peppermint | *Mentha × piperita* | monoterpene biosynthesis | mevalonate-independent pathway | isoprenoids

Isoprenoids are a large and structurally diverse family of compounds that play essential roles in plants as hormones, photosynthetic pigments, electron carriers, and membrane components and that also serve in communication and defense (1). Although isoprenoids are universally synthesized through condensations of the five-carbon compound isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP), two distinct and independent biosynthetic routes to these precursors exist in plants. The cytosolic pathway to IPP (Fig. 1A) starts from acetyl-CoA and proceeds through the classical intermediate mevalonic acid to provide precursors for the biosynthesis of sesquiterpenes (C_{15}) and triterpenes (C_{30}) (2). The plastidial pathway (Fig. 1B) is initiated by the transketolase-type condensation of pyruvate (carbons 2 and 3) and glyceraldehyde-3-phosphate to 1-deoxyxylulose-5-phosphate (DXP), followed by the isomerization and reduction of this intermediate to 2-C-methylerythritol-4-phosphate, formation of the cytidine 5'-diphosphate derivative, phosphorylation at C2, and cyclization to 2-C-methylerythritol-2,4-cyclodiphosphate as the last defined step (3–6). This plastidial pathway provides precursors

for the biosynthesis of isoprene (C_5), monoterpenes (C_{10}), diterpenes (C_{20}), and tetraterpenes (C_{40}) (4, 7), and genes encoding each enzyme of the pathway, up to formation of the cyclic diphosphate, have been isolated from plants and from eubacteria in which the pathway also operates (8–19).

Transgenic manipulations of the mevalonate-independent (DXP) pathway in *Escherichia coli* have indicated that IPP and DMAPP likely arise independently by branching of the pathway (20) and that overexpression of the first pathway gene, for DXP synthase (DXPS), increases carotenoid and ubiquinone biosynthesis (21, 22); manipulation of the mevalonate pathway that operates in yeast also results in increased carotenoid production (23). Studies on the results of overexpression and underexpression of DXPS in *Arabidopsis* have recently indicated that this enzyme catalyzes a slow step in the mevalonate-independent pathway to plastidial isoprenoids (chlorophylls and carotenoids) (24), and considerable literature exists on the transgenic alteration of hydroxymethylglutaryl CoA reductase in plants and the influence on cytosolic isoprenoid production (sesquiterpene phytoalexins and phytosterols); however, the roles of the various reductase isoforms in differentially regulating the mevalonate pathway are not fully clear (25–28). The control of flux through each pathway of isoprenoid biosynthesis in plants, in which both mevalonate and mevalonate-independent (DXP) pathways operate, and the level and means of interaction between the two pathways are of considerable interest in the context of both primary and secondary plant metabolism.

Monoterpenes comprise the major components of the essential oils of the mint family (Lamiaceae), including peppermint (*Mentha × piperita*), which has been developed as a model system for the study of monoterpene metabolism. Peppermint oil is chemically complex, and the biosynthetic pathway leading to the major monoterpene component (–)-menthol (Fig. 2) involves a broad range of representative reaction types of terpenoid metabolism (e.g., cyclization, hydroxylation, redox transformations) (29). Monoterpene biosynthesis in mint is specifically localized to the glandular trichomes (30) and originates in the leucoplasts of the secretory cells of these highly specialized nonphotosynthetic epidermal structures (31). During the brief but intense period of secretory activity (32, 33), monoterpene biosynthesis is driven by plastidial supply of IPP and DMAPP via the DXP pathway; the cytosolic mevalonate pathway is also inactive at this stage of oil gland development (34). It is of interest to determine whether flux through the mevalonate-independent pathway is

Abbreviations: DMAPP, dimethylallyl diphosphate; DXP(S), deoxyxylulose phosphate (synthase); DXR, DXP reductoisomerase; IPP, isopentenyl diphosphate; MFS, menthofuran synthase; NPT, neomycin phosphotransferase; WT, wild type.

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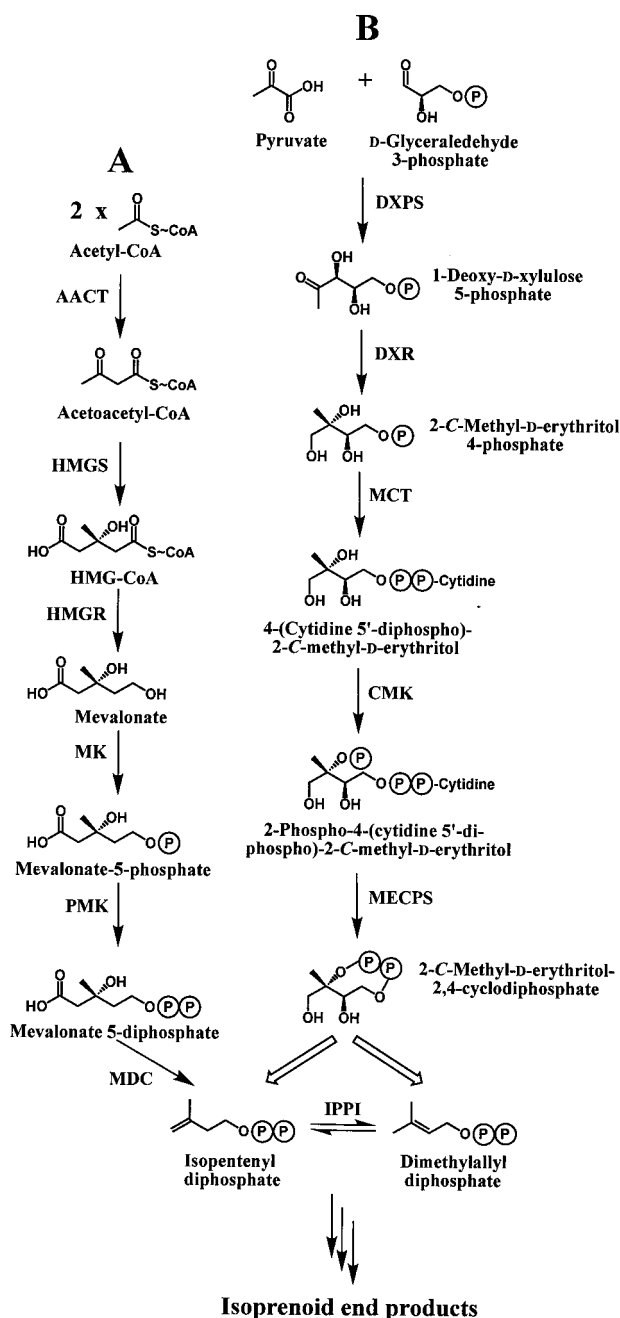


Fig. 1. Biosynthesis of IPP and DMAPP via the mevalonate pathway (A) and the mevalonate-independent (DXP) pathway (B). The indicated enzymes are: AACT, acetyl-CoA/acetyl-CoA C-acetyltransferase; HMGS, 3-hydroxy-3-methylglutaryl-CoA synthase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; MK, mevalonate kinase; PMK, phosphomevalonate kinase; MDC, mevalonate-5-diphosphate decarboxylase; DXPS, 1-deoxyxylulose-5-phosphate synthase; DXR, 1-deoxyxylulose-5-phosphate reductoisomerase; MCT, 2-C-methylerythritol-4-phosphate (MEP) cytidyltransferase; CMK, 4-(cytidine-5'-diphospho)-2-C-methylerythritol kinase; MECPS, 2-C-methylerythritol-2,4-cyclodiphosphate synthase; and IPP isomerase (IPPI). The circled P denotes the phosphate moiety. The large open arrows indicate several as-yet-identified steps. The pathway may give rise to IPP and DMAPP independently (20) of the interconversion catalyzed by IPPI.

limiting during the period of very rapid terpenoid biosynthesis by manipulating this route for precursor supply. Such a finding could have important implications for production of the essential oils and other terpenoids of commercial significance (35).

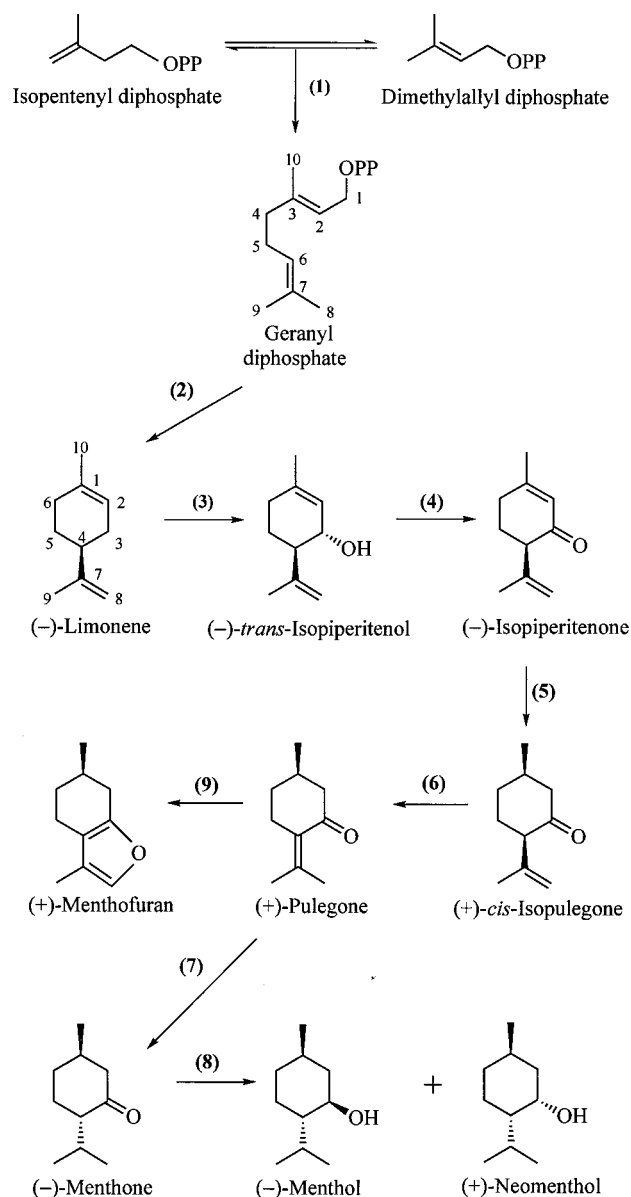


Fig. 2. The principal pathway for monoterpene biosynthesis in peppermint. The responsible enzymes are: 1) geranyl diphosphate synthase; 2) (-)-limonene synthase; 3) cytochrome P450 (-)-limonene-3-hydroxylase; 4) (-)-trans-isopiperitenol dehydrogenase; 5) (-)-isopiperitenone reductase; 6) (+)-cis-isopulegone isomerase; 7) (+)-pulegone reductase; 8) (-)-menthone reductase; and 9) cytochrome P450 (+)-MFS. The circled P denotes the phosphate moiety.

Because DXP is an intermediate not only for IPP and DMAPP biosynthesis but also for the biosynthesis of thiamin and pyridoxol (36, 37), it is the conversion of DXP to methylerythritol phosphate (Fig. 1B), catalyzed by DXP reductoisomerase (DXR) (11), that represents the committed step in the production of IPP. In this paper, we report the transformation of peppermint with the homologous cDNA for DXR (12) under the control of a strong constitutive promoter and describe the influence of modified expression of this gene on essential oil production yield and mint physiology.

(+)-Menthofuran is an undesirable monoterpene component of peppermint that is derived from the α,β -unsaturated ketone (+)-pulegone (38) (Fig. 2); it contributes off-flavor to the isolated essential oil and promotes off-color on storage (39, 40).

The content of menthofuran can reach industrially unacceptable levels in plants raised under stressful environmental conditions (high temperature, drought, low light intensity) (41, 42), over which commercial mint growers have very limited control. A cDNA-encoding cytochrome P450 (+)-menthofuran synthase (MFS) [(+)-pulegone-9-hydroxylase] was recently isolated from peppermint (38), thus offering a direct, but heretofore unexplored, means for transgenic manipulation of menthofuran production. In this paper, we also report the transformation of peppermint with the antisense version of (+)-MFS (38) under the control of a strong constitutive promoter, and we describe the influence of decreased expression of this gene on the composition of the essential oil produced in stressed and unstressed plants.

Materials and Methods

Plant Material. Peppermint plants (the sterile hybrid *Mentha* × *piperita* L. cv. Black Mitcham) were propagated from rhizomes and stem cuttings in flats containing peat moss/pumice/sand (55:35:10, vol/vol/vol) and were grown under controlled conditions at 500–600 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ photosynthetically active radiation at plant height, with a 16-h photoperiod and a 26°C/15°C (day/night) temperature cycle (43). To induce moderate stress, which alters oil composition by increasing the levels of (+)-menthofuran and (+)-pulegone (41, 42), the photon flux density was reduced to 200–300 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, and the night temperature was increased to 21°C. All plants were watered and fertilized daily with a complete fertilizer (N/P/K, 20:20:20) plus iron chelate and micronutrients, and all flats were grown to complete confluence, then pruned and regrown to maturity (preflowering) before harvesting for oil analysis.

Vector Assembly and Plant Transformation. The parent vector pGAdexG/Nuclear Inclusion-b protein (NIB).L was provided by J. C. Carrington of the Institute of Biological Chemistry. This vector is derived from pGA482 (44) and contains a β -glucuronidase (GUS)-NIB gene fusion inserted between the CaMV tandem 35S promoter with duplicated enhancer and the *Agrobacterium* NOS transcriptional terminator. The GUS-NIB fusion was excised with *EcoRI*/*KpnI* and replaced by ligation with the DXR cDNA, which was amplified from the original clone (12) by using forward primer (5'-ACTGTCGAATTCATGGCTCTAAACTTGATGGC-3') and reverse primer (5'-ATCGCTGGTACCGCTCATACAGAGCAGGAC-3') to introduce the respective 5'-*EcoRI* site upstream of the start codon and 3'-*KpnI* site downstream of the stop codon. The coding region (antisense version) of the MFS cDNA (38) was amplified by PCR by using primers (5'-CGCCGCGAATTCTCAAGATTGACGTTGGAGTAGC-3') and (5'-CGCCGCGGTACCATGGCCGCTCTTCTAG-3') to generate an *EcoRI* site and a *KpnI* site at the respective 3'- and 5'-termini of the gene. The resulting gel-purified amplicon was digested with *EcoRI* and *KpnI* and ligated into similarly prepared and gel purified pGAdexG/NIB.L to replace the original GUS-NIB insert as before.

The sequence-verified constructs were electroporated into *Agrobacterium tumefaciens* strain EHA105 by using the MicroPulser (Bio-Rad) according to the manufacturer's protocol. A single transformant bearing each construct was isolated and grown to log phase in minimal medium (45) containing 50 mg of kanamycin L^{-1} and 30 mg of rifampicin L^{-1} , harvested by centrifugation, resuspended in minimal medium containing 0.2 mM acetosyringone, and used to infect peppermint leaf discs as previously described (46, 47). After regeneration by established protocols (46, 47), rooted plantlets were transferred to soil, acclimated, and then moved to the greenhouse and propagated as above.

RNA Isolation and Blot Analysis. Total RNA was extracted from immature (1–2 cm) and fully expanded (>4 cm) peppermint leaves by using the Trizol Reagent (GIBCO/BRL) according to the supplier's protocol. Ten micrograms of denatured RNA was separated by electrophoresis on a 1.2% agarose-formaldehyde gel and transferred to a Hybond-N nylon membrane (Amersham Pharmacia) by standard protocol (48). ^{32}P -labeled DNA probe, prepared by random priming of the cDNA encoding DXR, was used to detect the corresponding mRNA. Prehybridization was conducted at 65°C for 1 h in 0.5 ml/cm² of Rapid Hyb buffer (Amersham Pharmacia), followed by hybridization with the ^{32}P -labeled probe (8×10^6 cpm) under the same conditions for 2 h, and then washing in $4\times$ (15 min, room temperature), $2\times$ (15 min, 65°C), and $1\times$ (15 min, 65°C) SSC containing 0.1% SDS before exposure to Kodak X-Omat x-ray film overnight.

Enzyme Isolation and Assay. Soluble enzyme extracts from peppermint leaves (2–3 cm in length, 0.5 g) were prepared by a standard procedure (49). The resulting soluble enzyme fraction (8 ml) was then suspended with ceramic hydroxyapatite (Bio-Rad, 2 g matrix/8 ml extract) that had been prewashed and equilibrated with extraction buffer [20 mM potassium phosphate (pH 6.5)/10 mM sodium ascorbate/10 mM MgCl_2 /1 mM DTT]. The slurry was gently mixed for 1 h at 0–4°C to allow protein adsorption, and the matrix was then removed by centrifugation to provide a supernatant essentially free of phosphatase activity that interferes with the DXR assay and neomycin phosphotransferase (NPT) assay. The NPT assay followed an established literature procedure (50). The preparation of the substrate [$1\text{-}^{14}\text{C}$]DXP and the details of the radio-HPLC-based assay for DXR activity have been previously described (12).

Essential Oil Analysis. Confluent flats of transgenic mint or wild-type (WT) controls were grown to maturity (flower bud stage) and were individually harvested and frozen at –20°C. The frozen tissue was then manually crushed and mixed to ensure sample uniformity, and three 10-gram samples from each trial (large-stem fragments were excluded) were taken for simultaneous steam distillation–pentane extraction as previously described (43) by using (+)-camphor as an internal standard. One-microliter aliquots of the diluted distillate were analyzed for terpenoid content by gas chromatography (and coupled gas chromatography–mass spectrometry) as described elsewhere (43), and the products were quantified (in milligram/gram tissue fresh weight) by comparison of detector response with that of the internal standard.

Results and Discussion

The first step of the plastidial mevalonate-independent pathway for the production of isoprenoid precursors is catalyzed by DXPS (5, 6), which also supplies precursor (DXP; see Fig. 1) for the synthesis of thiamin and pyridoxol (36, 37). The second step of the pathway is catalyzed by DXR (for the conversion of DXP to methylerythritol phosphate; see Fig. 1), which is considered the committed step in the supply of terpenoid precursors (11) and thus a potential target for control of flux through this branch of the pathway. There have been no previous attempts to manipulate DXR or to evaluate the influence of this or any other gene of the mevalonate-independent pathway on the production yield of essential oil terpenes. A cDNA encoding DXR was isolated from peppermint (12); this 1,425-nt sequence encodes a preprotein bearing an N-terminal plastidial peptide that directs the enzyme to the plastids where the mevalonate-independent pathway operates. The mature enzyme comprises about 400 amino acid residues with a size of about 43.5 kDa, and it resembles other reductoisomerases of plant and eubacterial origin (51).

(+)-MFS was recently demonstrated to be a cytochrome P450 enzyme capable of hydroxylating the *syn* (C9)-methyl group of

(+)-pulegone, which leads to spontaneous intramolecular cyclization to the hemiketal and dehydration to the furan, to yield this commercially undesirable essential oil component (38). An abundant cytochrome P450 clone from a peppermint oil gland cell cDNA library (52) was functionally expressed in *Saccharomyces cerevisiae* and *E. coli* and shown to encode MFS (38), thus offering a transgenic means for control of menthofuran production. The full-length cDNA contains 1,479 nucleotides and encodes a protein of 493-aa residues of molecular weight 55,360, which bears a typical N-terminal membrane insertion sequence and all of the anticipated primary structural elements of a cytochrome P450.

Preparation and Evaluation of Transformed Plants. Genetic transformation of peppermint was accomplished by an established protocol by using *A. tumefaciens* strain EHA105 (46, 47) and a binary vector pGA482 (44) containing *npt* and the full-length (sense) *dxr* construct (12) or the resistance gene and the antisense version of *mfs* (38). Subsequent regeneration and selection from leaf disks transformed with the sense version of *dxr* yielded 57 kanamycin-resistant plants, and of leaf disks transformed with the antisense version of *mfs* yielded 19 kanamycin-resistant plants. Gene transfer in both cases was confirmed directly by assay of leaf extracts for expression of the selectable marker (*npt*) (50), and all NPT-positive plants were propagated for further analysis.

All 19 of the verified transformants bearing the antisense MFS cDNA and most transgenic plants transformed with the DXR (sense) cDNA (42 plants designated the TI group) were indistinguishable from WT plants. In the population of *dxr* transformants, 11 plants (designated the TIIA group) did not develop normal pigmentation; instead, the leaves appeared uniformly lighter green, suggesting that chlorophyll synthesis was impaired. These plants grew more slowly and produced less biomass than did WT. A third group of *dxr*-transformants (four plants designated TIIB) also lacked normal pigmentation in a mosaic pattern.

To determine whether the phenotypic variation observed in the *dxr*-transformants correlated with the expression pattern of the *dxr* transgene, total leaf RNA was isolated for Northern blot analysis by using the DXR cDNA as probe. The results showed that DXR mRNA was strongly expressed in young leaves of WT plants and TI plants (WT appearance) and was easily detected in mosaic plants but not in leaves in which *dxr* was apparently cosuppressed (Fig. 3A). In fully expanded leaves, the DXR message was not detectable in WT (or cosuppressed) plants; however, the level of this transcript increased significantly in proportion to total RNA in TI transgenics and was also observed in TIIB mosaic plants (Fig. 3B). Because the DXP pathway operates in plastids to supply precursor for the biosynthesis of essential metabolites, such as chlorophyll (4, 6), the high-level expression of *dxr* in young leaves is not surprising. As leaves mature, however, the expression levels of many genes, including *dxr*, might be expected to decrease. In transgenic plants, the DXR cDNA was constitutively expressed under control of the CaMV 35S promoter (53). Thus, as leaves mature and many genes are developmentally silenced, the CaMV 35S promoter remains active, resulting in an increase in the proportion of transgene DXR mRNA to total leaf RNA and, as a consequence, may maintain DXP pathway function. In the TIIA group, DXR message was not detectably expressed in immature or fully expanded leaves, as determined by Northern blot analysis (Fig. 3), indicating that the *dxr* gene was cosuppressed (54–56) in these plants. Such down-regulation of *dxr* would very likely compromise chlorophyll biosynthesis and result in the phenotypic lack of pigmentation observed.

To assess DXR activity in transgenic plants, DXR assays were performed with soluble protein extracts from developing leaves of plants in each phenotypic category. These results correlated

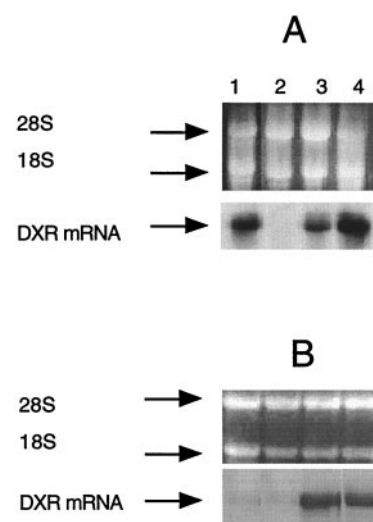


Fig. 3. Measured mRNA levels for DXR in immature (A) and fully expanded (B) leaves of WT and transgenic peppermint plants. Total leaf RNA was isolated, separated on a denaturing agarose gel (10 μ g/lane), blotted, hybridized to the radiolabeled DXR cDNA as probe, and exposed to film (Lower). The indicated lanes correspond to: Lane 1, WT plant; Lane 2, transgenic cosuppressed plant; Lane 3, transgenic mosaic plant; and Lane 4, transgenic plant with WT appearance that overexpresses *dxr*. Upper illustrates ribosomal bands visualized with ethidium bromide that were used to verify loading of equal amounts of total RNA before transfer.

well with the Northern blot data, in that extracts of TI plants that over-expressed the DXR cDNA contained two to four times more DXR activity (on a $\text{nmol}\cdot\text{h}^{-1}\cdot\text{mg}$ of protein $^{-1}$ basis) than did the corresponding extracts from WT plants. Conversely, DXR activity was not detected in extracts of plants in which *dxr* was seemingly cosuppressed, although at least low levels of DXR activity must have been present in these plants because they did grow, albeit slowly, and they were not albino.

Effects on Essential Oil Production and Composition. Because glandular trichome metabolism in mint is largely dedicated to monoterpene production driven by precursor supply from the plastidial DXP pathway (7, 12, 34), it was reasoned that alterations in pathway flux because of changes in *dxr* expression should be observable at the level of essential oil accumulation. Essential oil analysis of mint is easily accomplished by steam distillation of leaf tissue followed by gas chromatographic separation of components of the distillate and quantification by using an internal standard (43). These analytical results (Table 1) demonstrated that most plants in the TI group accumulated more oil than WT plants (up to nearly 50% increase in oil yield), whereas plants apparently cosuppressed for *dxr* (TIIA group) produced less oil than did control plants. These analyses further demonstrated that the composition of the essential oil of the transgenic plants was similar to WT in the majority of cases (55 plants). However, two plants produced a significantly different oil composition compared with WT and to the other transgenic plants. One plant (designated DXR16 of the TI group) accumulated higher quantities of menthofuran and pulegone (Fig. 2), whereas a second plant (DXR46 of the TI group) accumulated less pulegone and menthofuran, but more menthol, than did WT plants (Table 1). Additionally, plant DXR46 produced piperitone oxide to a level of about 5% of total oil; this compound was not detected in WT or other transgenic plants. The abnormal oil compositions of DXR16 and DXR46 plants are not consistent with those of other transgenic plants in their respective groups. Thus, it seems likely that these changes in oil profile are not caused by alterations in *dxr* expression but rather are the result of insertional effects of the transgene that

Table 1. Essential oil yield and composition of WT peppermint and selected transformants expressing the sense version of DXR reductoisomerase (DXR plants) and the antisense version of menthofuran synthase (MFS plants)

Plant	Oil yield (mg/g fresh weight)	Percentage					
		Limonene	Cineole	Menthone*	Menthofuran	Pulegone	Menthol
WT [†]	1.8	2.4	3.8	45.9	16.8	8.0	6.9
DXR6	2.6	2.0	3.4	45.0	15.7	6.1	12.7
DXR7	2.3	2.0	3.8	55.8	7.2	3.2	11.0
DXR8	2.4	1.9	3.9	45.0	15.5	5.7	12.6
DXR16	1.4	1.8	3.0	23.6	36.4	16.8	6.5
DXR32	2.6	2.2	3.9	46.1	12.5	5.7	13.9
DXR37	2.7	2.2	3.8	47.9	14.7	7.3	13.3
DXR38	2.6	2.0	4.6	50.7	13.7	5.3	13.2
DXR40	2.7	1.7	5.1	62.8	13.6	5.2	13.3
DXR44	2.4	1.9	3.3	38.6	15.0	6.2	11.0
DXR46	1.7	4.8	4.8	45.3	5.1	1.7	27.0
WT [‡]	2.3	1.9	4.6	64.0	5.0	2.0	8.5
MFS1	1.7	1.1	5.3	35.0	2.5	0.2	23.1
MFS3	1.4	1.7	5.8	63.7	2.5	0.7	12.7
MFS7	2.4	1.3	6.3	53.5	2.5	0.8	19.5
MFS15	1.8	1.8	4.0	65.2	3.2	1.3	10.0
WT [§]	1.7	2.3	4.3	60.2	13.9	7.8	4.0
MFS7	1.8	2.6	5.0	68.8	5.3	2.8	7.3

All measurements represent the averages of three replicates of two independent tissue samples, SE \pm 10%. Each group of transformants was compared to WT plants grown under the same conditions.

*Isomenthone is not included. The combination of menthone plus isomenthone generally constitutes 60–70% of the oil.

[†]This oil composition is typical of newly established plants raised under these moderate stress growth conditions.

[‡]This oil composition is typical of newly established plants raised under these unstressed growth conditions.

[§]This oil composition is typical of established plants raised under these moderate stress growth conditions.

serve, directly or indirectly, to down-regulate pulegone reductase (DXR16) and MFS (DXR46) (see Fig. 2).

In the case of peppermint plants transformed with the antisense version of *mfs*, most (15 plants) produced an oil of near average composition and yield compared with WT (data not shown). However, four of these plants (MFS1, 3, 7, and 15) accumulated 35–55% less (+)-menthofuran (and 40–60% less (+)-pulegone), and substantially more (–)-menthol, than WT controls (Table 1). Oil evaluation over a period of 6 months (four independent distillations and analyses) demonstrated that the MFS7 transgenic plant consistently produced an oil of comparable yield with lower levels of menthofuran and pulegone, and higher levels of menthol, than WT plants. This pattern of uncompromised oil yield and compositional modification persisted even when plants were grown under stress conditions (obtained by elevated night temperature combined with decreased photon flux during the daylight period) that are known to promote the production and accumulation of menthofuran and pulegone (41, 42) (Table 1). It is notable that peppermint plants transformed with *mfs* in antisense orientation (MFS1, 3, 7, and 15) produce an essential oil very similar in composition to the DXR46 plant transformed with the sense version of the reductoisomerase (Table 1), suggesting that the latter bears an insertion that inactivates the *mfs* gene to produce a similar oil compositional change.

Conclusions

The present results directed to the manipulation of *dxr* as the committed step of the mevalonate-independent pathway to terpenoids support previous findings (24, 57, 58) with *Arabidopsis* in which disruption of *dxps* (the *clal* gene encoding the first step of the mevalonate independent pathway) led to early arrest of chloroplast development and an albino phenotype. In the present instance, both essential oil and chlorophyll biosynthesis were impaired in the *dxr* cosuppressed plants, but it was clear from the visible phenotype and essential oil chemotype that

precursor supply from the DXP pathway was not entirely eliminated in these plants.

Transgenic up-regulation of *dxr*, as evidenced by Northern blot analyses and direct DXR enzyme assays, led to an increase in essential oil accumulation, a result that may be attributed to improved flux of precursors for monoterpene biosynthesis in the oil glands by the increased level or developmental duration of the DXP pathway. Either effect implies that DXR catalyzes a slow step of the mevalonate-independent pathway. It is notable that essential oil yield increases approaching 50% did not result in observable changes in the complex oil composition noted for most plants. This coupling of yield increase without compositional change indicates that the capacity for limonene production (and downstream biosynthetic steps; see Fig. 2) has not been exceeded and thereby suggests that additional rate-determining step(s) reside somewhere between DXR and limonene synthase (the first committed step of monoterpene biosynthesis).

Transgenic down-regulation of *mfs*, by the antisense approach, led to the anticipated decrease in oil content of (+)-menthofuran (without change in yield) but surprisingly did not increase (+)-pulegone content as might be expected via the decreased conversion of this ketone intermediate to (+)-menthofuran (see Fig. 2). Rather, a decrease in the oil content of both menthofuran and pulegone was observed in the transgenic antisense MFS plants (Table 1). This unusual observation is currently unexplained but nevertheless represents a favorable compositional change, because both menthofuran and pulegone are considered undesirable monoterpene components when present in peppermint essential oil at levels exceeding a few percent.

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